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Commentary

Ocular Neovascularization

Clarifying Complex Interactions

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Neovascularization within the eye contributes to visual loss in several ocular diseases, the most common of which are proliferative diabetic retinopathy, neovascular age-related macular degeneration, and retinopathy of prematurity. Together, these three diseases afflict persons in all stages of life from birth through late adulthood and account for most instances of legal blindness.

Retinopathy of prematurity (ROP) occurs in premature neonates. Normally, the retina becomes completely vascularized at full term. In the premature baby, the retina remains incompletely vascularized at the time of birth. Rather than continuing in a normal fashion, vasculogenesis in the premature neonatal retina becomes disrupted. Abnormal new proliferating vessels develop at the juncture of vascularized and avascular retina. These abnormal new vessels grow from the retina into the vitreous, resulting in hemorrhage and tractional detachment of the retina. Although laser ablation of avascular peripheral retina may halt the neovascular process if delivered in a timely and sufficient manner, some premature babies nevertheless go on to develop retinal detachment. Surgical methods for treating ROP-related retinal detachments in neonates have limited success at this time because of unique problems associated with this surgery, such as the small size of the eyes and the extremely firm vitreoretinal attachments in neonates.

Diabetic retinopathy is the leading cause of blindness in adults of working age. In persons with diabetes mellitus, retinal capillary occlusions develop, creating areas of ischemic retina. Retinal ischemia serves as a stimulus for neovascular proliferations that originate from pre-existing retinal venules at the optic disk or elsewhere in the retina posterior to the equator. Severe visual loss in proliferative diabetic retinopathy (PDR) results from vitreous hemorrhage and tractional retinal detachment. Again, laser treatment (panretinal photocoagulation to ischemic retina) may arrest the progression of neovascular proliferations in this disease but only if delivered in a timely and sufficiently intense manner. Some diabetic patients, either from lack of ophthalmic care or despite adequate laser treatment, go on to sustain severe visual loss secondary to PDR. Vitrectomy surgery can reduce but not eliminate severe visual loss in this disease.

Age-related macular degeneration is the leading cause of severe visual loss in persons over 65 years old. In contrast to ROP and PDR, in which neovascularization emanates from the retinal vasculature and extends into the vitreous cavity, AMD is associated with neovascularization originating from the choroidal vasculature and extending into the subretinal space. Choroidal neovascularization causes severe visual loss in AMD patients because it occurs in the macula, the area of retina responsible for central vision. The stimuli which lead to choroidal neovascularization are not understood. Laser ablation of the choroidal neovascularization may stabilize vision in selected patients. However, only 10% to 15% of patients with neovascular AMD have lesions judged to be appropriate for laser photocoagulation according to current criteria.

Retinopathy of prematurity, proliferative diabetic retinopathy, and neovascular age-related macular degeneration are but three of the ocular diseases which can produce visual loss secondary to neovascularization. Others include sickle cell retinopathy, retinal vein occlusion, and certain inflammatory diseases of the eye. These, however, account for a much smaller proportion of visual loss caused by ocular neovascularization. Additional treatments beyond laser photocoagulation and vitrectomy surgery are needed to improve outcomes in these patients. Pharmacological antiangiogenic therapy can potentially assist in prevention of the onset or progression of ocular neovascularization and is a current goal of many research laboratories and pharmaceutical companies.

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Factors Affecting Vasculogenesis and Angiogenesis

Development of pharmacological strategies for treating ocular neovascularization depends on our gaining a more thorough understanding of the processes involved in vasculogenesis (generation of primitive embryonic blood vessels from mesodermal cells called angioblasts) and angiogenesis (development of new vessels from pre-existing vessels).[1,2] Studies on such seemingly diverse topics as wound healing, tumor growth and metastasis, embryological development, and ophthalmic disease have all contributed to our understanding of basic mechanisms involved in new vessel formation.

Our current knowledge indicates that vasculogenesis and angiogenesis result from complex interactions between factors which either stimulate or inhibit endothelial cell differentiation, proliferation, migration, and maturation. Endothelial cells respond to regulatory proteins called growth factors that tend to be produced locally within the involved tissue and are secreted either by endothelial cells themselves or by neighboring cells.

Counterbalancing the effects of endothelial growth factors are naturally occurring endogenous angiogenesis inhibitors. In addition to responding to these soluble stimulatory and inhibitory factors, endothelial cells interact with and respond to changes in the extracellular matrix through cell surface receptors called adhesion molecules. Research in each of these areas has suggested possible means of pharmacological manipulation of endothelial cell behavior.

Growth Factors

A variety of endothelial cell growth factors have been identified including fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), placental growth factor (PGF), insulin-like growth factor 1 (IGF-1), and platelet-derived endothelial cell growth factor (PD-ECGF).[3,4,5] Of these, the FGF and VEGF families and their cell surface receptors have been most fully characterized. Our emphasis will be on basic FGF (bFGF, also known as FGF-2) because this is the growth factor under study in the article by Ozaki and co-authors in this issue.[6]

Fibroblast growth factor activity was discovered in 1940 when extracts from brain and pituitary were found to stimulate proliferation of cultured fibroblasts.[7] By the 1980s two forms of FGF, acidic FGF (aFGF) from brain and basic FGF (bFGF) from pituitary, had been purified using heparin-affinity chromatography.[8] Both forms (aFGF and bFGF) are single-chain proteins of approximately 140 amino acids. In addition to aFGF and bFGF, several other members of the FGF family now are known and a number of soluble and cell surface FGF receptors have been characterized.[9,10] Basic FGF is found in extracellular matrix (ECM) from which it can be released by ECM-degrading enzymes such as serine proteases and metalloproteases.[11] FGF receptors also have been localized in vascular extracellular matrix.[12]

Fibroblast growth factors are produced by a variety of cell types in culture including vascular endothelial cells, fibroblasts, smooth muscle cells, astrocytes, granulosa cells, adrenocortical cells, and retinal pigment epithelial cells, as well as a large number of malignant cell types. Fibroblast growth factors also function during embryonic development and wound healing.[13,14] Many of the cells which produce FGF, including vascular endothelial cells, also respond to this growth factor.

In vivo bFGF has angiogenic activity and is expressed in high levels by endothelial cells during tumor angiogenesis and vasculogenesis.[15] *In vitro* bFGF causes endothelial cell proliferation, protease production, and chemotaxis. In collagen matrices, bFGF enhances tube formation by endothelial cells.[16]

An unusual feature of aFGF and bFGF molecules is the absence of a secretory signaling sequence typical of proteins secreted by the endoplasmic reticulum–Golgi apparatus.[17] Apparently, bFGF is released from cells by a different mechanism.

Basic FGF is thought to exert both paracrine and autocrine influences on vascular endothelial cells. Paracrine angiogenic activity is illustrated by tumor angiogenesis where neoplastic cells which release bFGF form fast growing, highly vascularized tumors. Autocrine angiogenic activity has been demonstrated by experiments in which endogenously produced bFGF proved necessary for endothelial cell migration from a confluent monolayer into a denuded area of the culture plate from which a patch of cells was removed with a razor blade.[18] Release of bFGF by vascular endothelial cells also increases their production of plasminogen activator, yet another autocrine effect.[19,20] Therefore, tumor angiogenesis and other neovascular diseases may be initiated by stimuli which increase autocrine production of bFGF.

Evidence for Involvement of bFGF and VEGF in Ocular Neovascular Diseases

Fibroblast growth factor has been studied in relation to two ocular neovascular disease processes: retinal neovascularization in PDR and choroidal neovascularization in AMD. Vitreous samples from patients with proliferative diabetic retinopathy reveal elevated levels of bFGF compared to controls.[21,22] Choroidal neovascular membranes removed from human patients with AMD show evidence of FGF expression.[23,24]

In recent years, VEGF has captured the attention of many investigators involved with ocular neovascularization. The VEGF family of growth factors consists of dimeric glycoproteins which induce endothelial mitogenesis and increase vascular permeability.

Multiple lines of evidence suggest a role for VEGF in ocular neovascular diseases. For example, VEGF levels are increased in the vitreous of patients with proliferative diabetic retinopathy compared to the vitreous of nondiabetic subjects.[25] VEGF mRNA expression is also increased in a mouse model of oxygen-induced proliferative retinopathy.[26] Human choroidal fibroblasts and retinal pigment epithelial cells express low levels of

VEGF. On stimulation with phorbol esters (which activate protein kinase C), choroidal fibroblast VEGF production increases.[27] Surgically excised choroidal neovascular membranes from patients with AMD demonstrate immunohistochemical staining for VEGF and VEGF mRNA by *in situ* hybridization.[28]

Current evidence indicates that no single growth factor acts alone to cause ocular neovascularization. *In vitro* studies of bovine microvascular endothelial cells suggest a role for bFGF in regulating the activity of VEGF.[29] In culture, VEGF-induced angiogenesis by bovine microvascular endothelial cells was blocked by the addition of antibodies to bFGF. The disruption in angiogenesis was accompanied by failure of the bovine endothelial cells to produce plasminogen activator. This finding may be another illustration of the possible autocrine function of bFGF in angiogenesis and neovascularization. It also indicates interaction of bFGF with VEGF in the production of angiogenesis.

Endogenous Inhibitors of Angiogenesis

Growth factors which promote angiogenesis appear to be counterbalanced by endogenous compounds which inhibit angiogenesis. Examples of these natural inhibitors of angiogenesis include angiostatin, glioma-derived inhibitory factor, endostatin, and thrombospondin, which have been found in association with tumor-related angiogenesis.[30,31]

One of these angiogenesis inhibitors also has been studied in relation to ocular disease, thrombospondin-1. Thrombospondins are a family of proteins present in platelet granules and secreted by several cell types including tumor cells.[32] These proteins have varied effects on different cell types. Thrombospondins cause proliferation of fibroblasts and smooth muscle cells but inhibit proliferation of endothelial cells. There are at least five thrombospondins, of which thrombospondin-1 is most interesting with respect to endothelial cell function.

Thrombospondin-1 is produced by vascular endothelial cells, fibroblasts, smooth muscle cells, lens epithelium, corneal endothelium, and other cell types.[33,34] Thrombospondin-1 has been located in surgical specimens of fibrovascular membranes in patients with proliferative diabetic retinopathy.[35]

In vitro thrombospondin-1 inhibits endothelial cell proliferation and adhesion. *In vivo* it inhibits angiogenesis. Synthetic peptides containing sequences from thrombospondin-1 inhibit endothelial cell chemotaxis in response to bFGF.[36] Both bFGF and PDGF induce increased expression of thrombospondin indicating interaction between positive and negative angiogenic stimuli.

Interaction of Cell Adhesion Molecules with the Extracellular Matrix

An important class of cell adhesion molecules are the integrins.[37] Integrins are composed of noncovalently associated α and β chains. Multiple α and β subunits exist, and these can combine to produce a variety of heterodimeric integrins. Integrins can bind to an array of extracellular matrix (ECM) components including laminin, collagen, fibronectin, thrombospondin, fibrinogen, and vitronectin. The binding of an integrin to an ECM component sends an intracellular signal that initiates a variety of endothelial responses such as adhesion, migration, proliferation, and apoptosis. Experimental evidence indicates that integrins are involved in tumor angiogenesis and in ocular neovascular diseases.[38,39] Friedlander and colleagues demonstrated the dependence of bFGF-induced rabbit corneal and chick chorioallantoic membrane neovascularization on the integrin $\alpha_V\beta_3$. Their work also indicated that VEGF-induced neovascularization in these models depended on a different integrin, $\alpha_V\beta_5$.[40] Friedlander and colleagues also demonstrated $\alpha_V\beta_3$ in choroidal neovascular membranes from patients with AMD and ocular histoplasmosis syndrome.[41] Both $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins were present on fibrovascular membranes removed from patients with PDR.

Mouse Model of Proliferative Retinopathy

A description of the mouse model of oxygen-induced proliferative retinopathy is in order here because this is the model used by Ozaki and co-authors in this issue.[6] Animal models of oxygen-induced proliferative retinopathy were originally developed to study the pathogenesis of ROP. As early as 1954, Patz described retinal neovascularization in neonatal rats raised in a hyperoxic environment.[42] However, other investigators could not reproduce retinal neovascularization in this model and interest in the model waned for a number of years. Because of improved survival of low-birth-weight premature babies in recent years, the incidence of ROP has again increased. With this resurgence of ROP and with the extensive interest in neovascular ocular disease in general, interest in animal models of proliferative retinopathy has been renewed.

Penn and colleagues reported a model of preretinal neovascularization in neonatal rats exposed to periodically varying oxygen concentrations (40% oxygen alternating with 80% oxygen at 12-hour intervals for approximately 1 week followed by room air).[43] Preretinal neovascularization occurred in 66% of all neonatal rats treated in this manner.

In 1994 Smith and colleagues reported an improved method for inducing proliferative retinopathy in newborn mice.[44] Previous studies in newborn mice had demonstrated engorgement of the hyaloid vascular system in newborn mice exposed to hyperoxia.[45] Smith and colleagues reasoned that the oxygen-induced hyaloidopathy may have confused previous studies of neovascular retinopathy in mice. Therefore, they allowed their newborn mice to live in room air for the first week of life to permit the hyaloid vascular system to regress (as normally occurs). Then, on day 7, the newborn mice were placed into a 75% oxygen environment where they lived for 5 days. This was followed by return to room air. Smith and colleagues found that 100% of the neonatal mice

treated in this way developed preretinal neovascularization at the juncture between vascularized and avascular retina. No hyaloid vasculopathy developed.

Further work on the mouse model by the same group of investigators has clarified the behavior of retinal blood vessels and the role of VEGF in response to hyperoxia and hypoxia.[46] In normal mice at 7 days of development in room air, VEGF mRNA was present just anterior to the zone of developing retinal blood vessels. Exposure of 7-day-old mice to 75% oxygen for 24 hours caused irreversible vaso-obliteration and an 85% reduction in levels of VEGF mRNA. Injection of exogenous VEGF into the eyes of 7-day-old mice exposed to hyperoxia decreased the degree of retinal vaso-obliteration. On return of mice to room air after 5 days of hyperoxia, retinal VEGF mRNA levels increased by threefold during the first 12 hours of relative hypoxia induced by return to room air.[26] If hyperoxia-treated mice were returned to room air for 24 hours but then treated with 75% oxygen for the next 24 hours, the hypoxia-induced elevation of VEGF mRNA was prevented. Thus, VEGF plays a major role in supporting retinal angiogenesis in the newborn mouse, and the relative hypoxia produced by returning hyperoxia-treated newborn mice to room air acts as a stimulus for VEGF production in the retina.

In this issue Ozaki and colleagues report on their recreation of this model of oxygen-induced proliferative retinopathy in genetically altered neonatal mice.[6] Mice deficient in bFGF (FGF-2 knockout mice) developed retinal neovascularization identical to that of wild-type mice. In addition, mice with bFGF overexpression (FGF-2 transgenic mice) developed retinal neovascularization identical to that of wild-type mice. As the authors point out in their discussion, the importance of bFGF to *in vivo* embryonic vasculogenesis and to ocular neovascularization may be less than previously assumed.

Transgenic Mice in the Study of Vasculogenesis and Angiogenesis

Genetically altered mice have proved invaluable in many areas of the biological sciences including the study of vasculogenesis and angiogenesis. In particular, experiments with gene knockout mice as described by Ozaki and co-authors in this issue have permitted *in vivo* observations and documentation of the effect of the absence of specific growth factors or growth factor receptors on the organism.[6]

For example, gene knockout mice have been created for endothelial cell transmembrane tyrosine kinases which function as receptors for the VEGF and angiopoietin families of growth factors.[47] Two receptors for VEGF are named Kdr/Flk-1 (kinase insert domain containing receptor/fetal liver kinase-1) and Flt-1 (fms-like tyrosine kinase-1). Two other endothelial tyrosine kinase receptors whose ligand appears to be angiopoietin are named Tie-1 and Tie-2.[48] Specific gene knockout mice have been created for all four of these tyrosine kinase transmembrane receptors.[49,50,51] The resulting phenotypes reveal something of the function of each growth factor and receptor.

Mice lacking either VEGF receptor die at about embryonic day 8.5 from vascular defects. Mice lacking Flt-1 receptors have endothelial cells which proliferate and migrate but fail to undergo tube formation. These mice develop a normal hematopoietic system. However, Kdr/Flk-1 knockout mice lack both endothelial cells and hematopoietic cells.

Tie knockout mice live somewhat longer than do mice lacking Kdr/Flk-1 and Flt-1. Tie-2 knockout mice live until roughly embryonic day 10. In these mice endothelial cells are present and even organize into tubes. However, the endothelial cells lack proper attachment to basement membranes and the resulting vessels remain immature and fail to form branching networks. Tie-1 knockout mice live longer than do Tie-2 knockout mice and die anywhere from embryonic day 14 to birth from hemorrhage and edema. This finding suggests that Tie-1 affects vascular permeability.

Transgenic mice that overexpress various growth factors also provide valuable information. For example, Okamoto and colleagues studied the effect of VEGF overexpression in mice transgenic for the human VEGF gene.[52] Retinal neovascularization in these transgenic mice emanates from the deep retinal capillary bed and extends into the subretinal space. Although this process does not duplicate PDR or neovascular AMD exactly, it may prove to be a useful model for the study of angiogenesis and its inhibitors.

The report by Ozaki and colleagues in this issue documents the authors' use of bFGF knockout mice and transgenic mice that overexpress bFGF to isolate the role of bFGF in angiogenesis in the oxygen-induced model of proliferative retinopathy.[6] Other methods to study this question could have been used, such as *in situ* hybridization to study bFGF mRNA production, injection of antibodies to inactivate bFGF or its receptor, or intraocular injection of bFGF. A potential problem with studies using neutralizing antibodies to bFGF is the possibility of the antibody interacting with another uncharacterized antigen in the eye similar to bFGF that also contributes to the process under study, angiogenesis. Use of knockout mice and transgenic mice avoids this problem of cross-reaction and provides a specific, convincing method for studying the effects of bFGF absence or overabundance on angiogenesis.

Because of bFGF-extracellular matrix interactions and because of the possible autocrine role of bFGF, studies based on injection of bFGF into the vitreous cavity may not have direct bearing on the true role of bFGF during *in vivo* angiogenesis. *In vivo* bFGF binds strongly to the extracellular matrix and becomes mobilized by degradation of the matrix by proteolytic enzymes. Simple injection of bFGF into the extracelluar fluid of the eye may not represent the situation of bFGF overproduction as well as would the transgenic mouse model in which bFGF presumably would interact with the extracellular matrix in a more natural fashion.

The study by Ozaki et al adds to our understanding of the role of bFGF in a mouse model of oxygen-induced

proliferative retinopathy. No apparent difference was noted among the knockout mice, the transgenic mice overexpressing bFGF, and the wild-type mice. The retina in all three types of mice behaved the same when exposed to hyperoxia, and there was no difference in the proliferative retinopathy that developed. However, we cannot generalize these findings to other species or to other types of retinal and choroidal neovascularization. The specific role of bFGF and its interaction with positive and negative angiogenic factors in human vasculogenesis and angiogenesis remain to be clarified.

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